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Genetic diversity of dog rose (*Rosa canina* L.) using ISSR markers

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Abstract

Dog rose is one of the most important species in Rosaceae family used as a medicinal plant and a rootstock for ornamental roses. This species is native to Iran, therefore, identification of indigenous genotypes of this species is important for genetic preservation or breeding purposes. Genetic diversity estimation of plant materials is one of the important pre-breeding activities in breeding crops. In this study, genetic variation of 23 genotypes of *R. canina* was investigated using fifteen ISSR markers. The genotypes were collected from three regions of Ardabil province (Namin, Nir and Khalkhal). The results showed that all primers generated clear and consistent polymorphic bands (77% polymorphism) but, ISSR-15 revealed a high numbers of polymorphic bands and was superior to other markers. Also, the ISSR-15 produced the highest number of polymorphic bands with seven scorable bands, while the UBC-823 and UBC-825 markers had the least number of bands (3 bands). The clustering pattern of genotypes was related to geographic regions. The genotypes from Khalkhal region were separated from other genotypes (Nir and Namin genotypes) in cluster analysis. The results of the current study indicated that the ISSR markers separated genotypes based on geographic region. The best way to select parents is to use genotypes with high genetic distances. Therefore, we determined the genetic distance among genotypes. According to the results, ISSR is an efficient marker system that can provide excellent information among *R. canina* genotypes. Finally, the obtained results indicated that the *R. canina* genotypes investigated in this research have a wide genetic diversity.

Key words: Genetic distance, ISSR, Molecular markers and *Rosa canina*.

INTRODUCTION

The genus Rosa represents one of the largest genera in Rosaceae family, comprising of more than 100 terrestrial species, distributed across the temperate and sub-tropical regions of the northern hemisphere (Samai *et al.,* 2010) specifically in Europe, Asia, the Middle East and North America (Nilsson, 1997). Dog rose with the scientific name *Rosa canina* L. is an important perennial shrub used as garden shrub rootstock, ornamental rose and medicinal plant. It has hermaphrodite flowers and is pollinated by insects. Fruits of *Rosa canina* called 'rose hip' are rich in bioactive compounds including vitamin C, carotenoids, tocopherol, phenolic acid, flavonoids, tannin, pectin, organic acids and essential oil (Gao *et al.,* 2000). They are also used as medicinal supplements, reducing the risk of cardiovascular diseases, different forms of cancer and have an anti-inflammatory effect (Tabaei-Aghdaei *et al.,* 2007). Selection of disease and drought tolerant rootstock is important for commercial roses (Demir and Ozcan, 2001). The dog rose possesses a unique meiotic and reproductive system consisting of a heterogamous meiosis with tetraploid egg cells and haploid pollen. Outbreeding leads to forming a permanent pentaploid organism, which are matroclinal in characters due to the differential contribution of maternal (80%) and paternal genomes (20%) (Winther *et al.,* 2016).

DNA based molecular markers have shown to be promising for the assessment of genetic diversity. The polymerase chain reaction (PCR)-based marker techniques, inter-simple sequence repeats (ISSR) are easy to use and widespread markers (Vijayan, 2005). Molecular markers have been developed to study genetic diversity in plants. Molecular markers have proven to be important in increasing selection efficiency; compared to conventional, morphologically based methods. They provide an easy and precise access to the genetic variability and they determine polymorphism at the DNA level without environmental interferences (do Val1 *et al.,* 2012).The utility of molecular tools for evolutionary studies arises from the insensitivity of the genetic markers to environmental factors (Hannachi *et al.,* 2010). Inter simple sequence repeat (ISSR)-PCR is a technique originated from microsatellite regions and uses microsatellite sequences as primers (16-18 bp) in a polymerase chain reaction to generate multi locus markers. ISSR-PCR gives multilocus patterns which are very reproducible, abundant and polymorphic in plant genomes. ISSR markers are useful in studies on genetic diversity, phylogeny, gene tagging and evolutionary biology (Reddy *et al.,* 2002).

Dog rose is one of the important rose species in Iran. In the distant past it was considered for its nutritional, medicinal and commercial value (Rahnavard *et al.,* 2013). Despite its long history, there is not enough information about genetic diversity of this plant. In the present study, an analysis of polymorphism among genotypes of *Rosa canina* was undertaken by ISSR markers. This will enable researchers to determine genetic groups or clusters to establish breeding programs encompassing the genetic diversity.

MATERIALS AND METHODS

Plant materials

Twenty-three genotypes of *R. canina* belonging to 3 regions from Ardabil province of Iran were collected in 2017 (Table 1).

DNA extraction

Genomic DNA was extracted from 100 mg of fresh mature leaves by a modified CTAB extraction method (Doyle and Doyle, 1990). The leaves were ground by liquid nitrogen in a sterile prechilled pestle and mortar. After the addition of 1 ml of extraction buffer [100 mM Tris-HCl, 2 M NaCl, 20 mM EDTA, 2% (w/v) PVP, pH 8], the mixture was homogenized and incubated at 65 ˚C in a water bath for 60 min and mixed thoroughly

Table 1. Geographical coordinates of the three different studied areas from Iran.

Numbers	Regions	Latitude	Longitude	Height (m)
1	N1	38° 01' 25" N	47° 00' 49" E	1620
2	N2	38° 02' 17" N	47° 58' 56" E	1650
3	N3	38° 02' 44" N	47° 57" 33" E	1701
4	N4	38° 04' 12" N	47° 57" 33" E	1750
5	N5	38° 03' 43" N	47° 54" 49" E	1570
6	N6	38° 01' 54" N	47° 04' 20" E	1800
7	Na1	38° 27' 00" N	48° 34' 06" E	1536
8	Na2	38° 27' 11" N	48° 34' 12" E	1580
9	Na3	38° 27' 24" N	48° 34' 34" E	1585
10	Na4	38° 27' 25" N	48° 34' 14" E	1600
11	Na5	38° 27' 32" N	48° 34' 20" E	1450
12	Na6	38° 27' 44" N	48° 34' 16" E	1650
13	Na7	38° 27' 39" N	48° 34' 37" E	1505
14	Na8	38° 27' 26" N	48° 34' 45" E	1700
15	Na9	38° 26' 45" N	48° 34' 05" E	1670
16	Na10	38° 27' 36" N	48° 33' 59" E	1605
17	Na11	38° 27' 17" N	48° 34' 04" E	1550
18	Na12	38° 27' 05" N	48° 33' 48" E	1500
19	Kh1	37° 17' 39" N	48° 31' 28" E	1850
20	Kh2	37° 37' 39" N	48° 30' 58" E	1900
21	Kh3	37° 38' 23" N	48° 30' 24" E	1890
22	Kh4	37° 38' 49" N	48° 30' 24" E	1820
23	Kh5	37° 39' 45" N	48° 30' 20" E	1930

N: Nir, Na: Namin, Kh: Khalkhal three regions from Ardabil province.

by a vortex. When the tubes were cooled to room temperature, an equal volume of 24:1 (v/v) mix of chloroform: isoamyl alcohol was added and shaken vigorously to form an emulsion. After centrifugation at 11,000 g for 20 min, the supernatant was separated and transferred into a new tube and mixed with 0.7 (v/v) volume of cold isopropanol (-20 ˚C) centrifuged again at 10,000 g for 5 min and the upper aqueous phase was separated. The precipitated DNA was washed with an equal volume of 70% (v/v) ethanol. Then pellets were dried and dissolved in 0.2 ml of double-distilled water. DNA quality was assessed by spectrophotometry and 0.8% agarose gel electrophoresis.

Inter-simple Sequence Repeats (ISSR) Molecular Fingerprinting Markers

Fifteen Inter-simple Sequence Repeats (ISSR) primers were chosen for amplifying the DNA (Table 2). The choice was based on the degree of polymorphism, as well as reproducibility of the amplified DNA fragments. DNA amplification was carried out in 10 µl reactions. The 10 µl volume PCR reactions contained 20 ng of genomic DNA, 5 µl of a PCR kit (Sigma, St. Louis, MO, USA), 1.1 µl of primer, and 2.5 µl of double distilled water. The amplification was performed by using Q-cycler thermocycler (HainLifescience, UK). The temperature profile consisted of an initial 5 min denaturation step at 94 ˚C, followed by 35 cycles, each had a denaturing step at 94 ˚C for 45 s, primer annealing at 52 ˚C for 30 s (depending on the primers) and extension step at 72 ˚C for 1 min. The final elongation step was set at 72 ˚C for 10 min. Amplification products were separated on a 1.5% agarose gel stained with fluorescent dye (4 µg/ml) in 1×TBE buffer. The ISSR bands were visualized under UV light and photographed with a digital camera. DNA molecular size marker (100 bp) (New England Bio Labs, USA) was used for the gel electrophoresis.

Data analysis

The observed bands on the gel were scored as present (1) or absent (0) for each entry. Very faint and non-reproducible bands were omitted from scoring. The genetic parameters such as; number of scored bands (NSB), number of polymorphic bands (NPB), observed number of alleles (Na), effective number of alleles (Ne) (Kimura and Crow, 1964), Nei's (1987) gene diversity (H), and Shannon's Information index (I) (Lewontin,1972) was calculated by PopGene program version 1.31 (Yeh *et al.,* 1999). Cluster analysis was performed by WARD (minimum spherical cluster) dissimilarity index using Windows (DARwin5) software (Perrier and Jacquemoud-Collet, 2006). For the population structure, a model analysis was fulfilled to infer the genetic structure and to clarify the number of sub-populations using the software STRUCTURE (version 2.3.4) (Pritchard *et al.,* 2000). The algorithm structure was run using ten independent replicate runs per *K* value from 1 to 10. Each run involved a burning period of 50,000 iterations. The number of supposed populations (K) was set from one to ten. The run with the maximum likelihood was employed to set genotypes into subpopulations.

Table 2. Selected primers for ISSR analyses in *Rosa canina*.

ISSR markers	Repeat motif (5'-3')	Ta (˚C)	Reference
UBC807	(AG)8 T	50	Bahmani *et al.,* 2015
UBC810	(GA)8 T	50	Brake *et al.,* 2014
UBC814	(CT)8 A	50	Brake *et al.,* 2014
UBC823	(TC)8 C	52	Bahmani *et al.*, 2015
UBC860	(TG)8 A	50	Brake *et al.,* 2014
UBC817	(CA)8 A	50	Pivoriene *et al.,* 2008
UBC809	(AG)8 G	48	Brake *et al.,* 2014
UBC825	(AC)8 T	51	Brake *et al.,* 2014
ISSR 10	(GACA)4	48.2	Brake *et al.,* 2014
ISSR 11	(GAC)5	51.6	Brake *et al.,* 2014
ISSR 12	(GA)6 CC	44	Brake *et al.,* 2014
ISSR 13	(CT)8 AC	54	Pivoriene *et al.,* 2008
ISSR 14	(ATG)6	47	Pivoriene *et al.,* 2008
ISSR 15	(ACTC)4	56	Brake *et al.,* 2014
ISSR 17	(CA)8 AG	54	Brake *et al.,* 2014

A, T, C and G: Nitrogenous bases, Ta: Temperature annealing.

RESULTS AND DISCUSSION

All primers generated clear and consistent polymorphic bands (Figure 1). A total of 58 reproducible amplified products were obtained, among which 45 bands were polymorphic (77% polymorphism). The number of DNA amplified fragments varied from 3 to 8, depending on the primer, with average of 3.8 per primer. The amplified fragment size ranged from 100 to 1200 bp. The ISSR-15 marker produced the highest NPB with 7 bands while the UBC-823 and UBC-825 markers had the least number of bands with 3 bands. Also, the highest and lowest percentages of polymorphic bands were observed for ISSR-15 and ISSR-17 markers (75 and 68%), as well as the UBC-823 marker showed the lowest polymorphism percentage (22%). In addition, the average Polymorphism Information Content (PIC) was 42.6. Parameters such as PIC value have been used increasingly for assessing the informative potential of ISSR markers (Gomes *et al.,* 2009). These values can range from 0 for monomorphic markers to 1 (Thimmappaiah *et al.,* 2009). The PIC value had a major role for comparing different markers in terms of their distinction powers. The high values of this parameter exhibited a high polymorphism; therefore, high PIC values are useful to separate genotypes with close relationship (Thimmappaiah *et al.,* 2009). This study also, Na, Ne, H, and I index were calculated, which had an average of 7.53, 7.07, 0.24 and 0.41, respectively. Shannon-index ranged from 0.18 to 0.66. The shannon index is analogue to genetic diversity, therefore, if the former is large; the latter will be large as well. Among these markers, ISSR-14, ISSR-15, and ISSR17 with the greater shannon information index (0.67, 0.66 and 0.65, respectively) exhibited more genetic diversity (Table 3) Mirzai *et al.* (2015) examined rose species by ten ISSR markers which amplified 81 bands and showed 86% polymorphism. Also, according to other results on genetic diversity such as; Samie *et al.* (2010) and Khosroushahi *et al.* (2014) and the results obtained from this research, it is clear that rose species have a high genetic variation in Iran.

Cluster analysis

The *R. canina* genotypes were separated into two major clusters revealed by WARD dendrogram (Figure 2). The first cluster (A) contained 19 genotypes and was subdivided into two subgroups. The first one (A) included 16 and second subgroup included three genotypes. The rest of genotypes constituted second main group (B) which comprised of four genotypes (19. 21, 22 and 23). According to Figure 2, most of the 19 genotypes of the first group belonged to Namin and Nir regions; which were not geographically distant. However, the genotypes from the Khalkhal region were well separated from the rest of the genotypes and separated into distinct groups. Results of the current study suggest that geographical distance has been effective in separating the genetic treasury of genotypes and ecological condition could have caused genetic variation. In this study the genetic relationships based on ISSR analysis supported geographical distance indicating that ISSR is an efficient marker system that can provide proper information on *R. canina* genotypes. Jurgens *et al.* (2009) investigated the genetic differentiation of *R. canina* in Brandenburg (Germany). The 55 genotypes were grouped in twelve subclusters. They mentioned that due to the outcrossing, polyploidy and seed dispersal system, the high genetic variation was observed within *R. canina* populations. Breeding system, seed dispersal, life form, and geographic distribution are decisive factors for determining the level of genetic variation and its

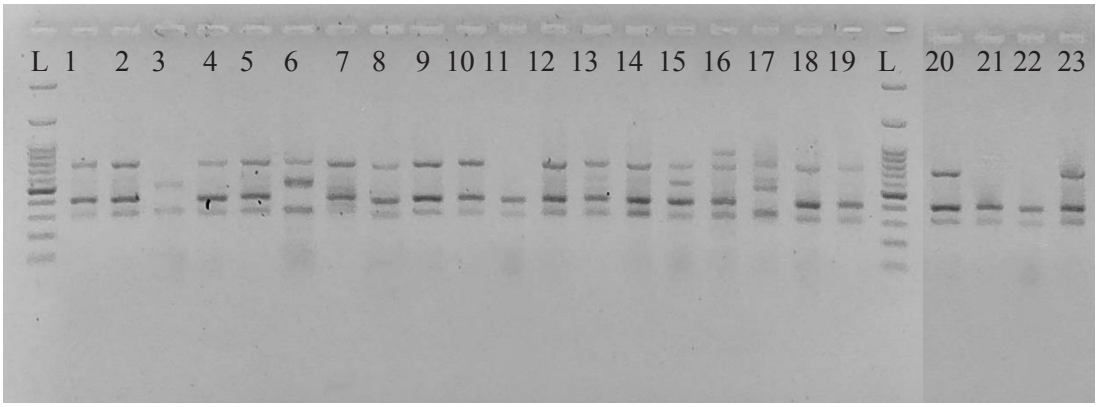

Figure 1. ISSR profiles of 23 genotypes using UBC810 ISSR primer. L=100 bp DNA size marker.

partitioning among populations. Rosa species are considered to be outcrossing species, but information about their outcrossing rates is limited (Jurgens *et al.,* 2007). With crossing experiments, Wissemann and Hellwig (1997) revealed crosspollination within species as the main reproduction form of *R. canina* producing the majority of seeds. In *Rosa rugosa*, 62.4% of fruit set occurred after cross-pollination (Ueda *et al.,* 1996), and in the study of Debener *et al.* (2003) on gene flow in self-incompatible roses, all roses were pollinated. This suggests high outcrossing rates in roses, and our results support this conclusion.

Table 3. The information obtained with ISSR markers in *R. canina*.

ISSR markers	NSB	NPB	Na	Ne	H	I	PIC value
UBC807	3	2	6	5.8	0.14	0.35	24
UBC810	5	4	10	9.2	0.44	0.63	64
UBC814	3	2	6	4.5	0.12	0.21	19
UBC823	3	2	5	4.6	0.09	0.18	22
UBC860	4	2	8	8.42	0.30	0.47	50
UBC817	3	2	6	5.6	0.23	0.32	35
UBC809	3	2	6	5.4	0.26	0.41	43
UBC825	3	2	5	4.8	0.12	0.26	25
ISSR 10	3	3	6	5.9	0.15	0.30	35
ISSR 11	4	4	7	6.6	0.19	0.44	46
ISSR 12	3	2	6	5.6	0.10	0.28	33
ISSR 13	3	2	6	5.4	0.20	0.36	35
ISSR 14	5	5	10	9.6	0.47	0.67	65
ISSR 15	8	7	16	15.3	0.46	0.66	75
ISSR 17	5	4	10	9.44	0.45	0.65	68
Mean	3.8	3	7.53	7.07	0.24	0.41	42.6

NSB: Number of scored bands, NPB: Number of polymorphic bands, Na: Observed number of alleles, Ne: Effective number of alleles, H: Gene diversity, I: Shannon's Information index, PIC: Polymorphic information content.

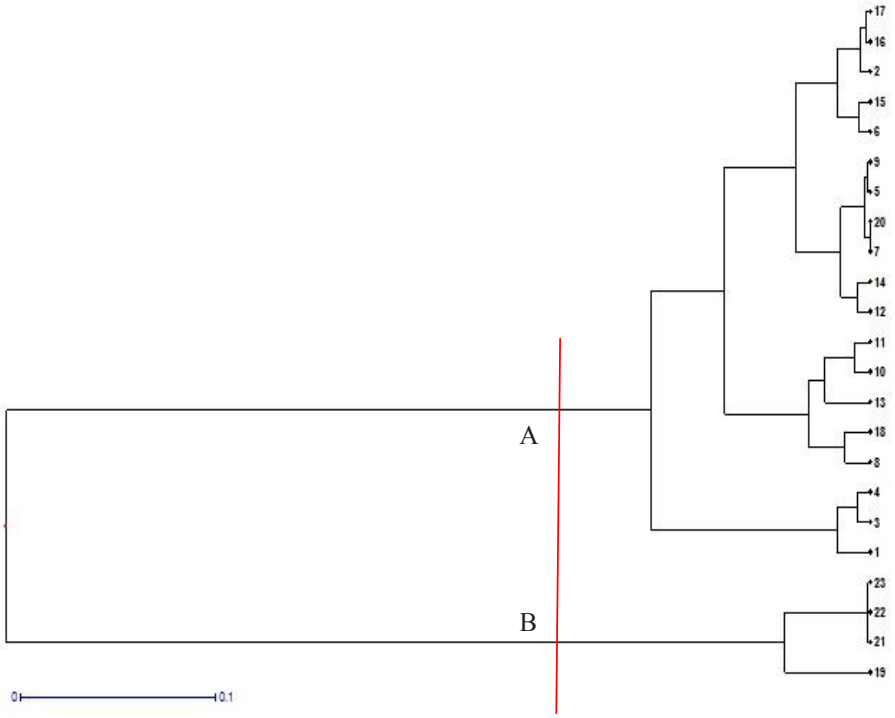

Figure 2. Dendrogram of 23 *R.canina* genotypes drawn based on ISSR markers according to Dice's similarity coefficient and WARD method.

Genetic structure

To determine the genetic structure in *R. canina* germplasm, a model-based analysis was performed using STRUCTURE 2.3.4 (Pritchard *et al.,* 2000). Based on the highest DK (Delta K) obtained from structure harvester, *K*=2 appeared to be the best model for *R. canina* genetic structure (Figure 3). According to the *K*=2 model, *R. canina* germplasm was structured into two gene pools. Most of the *R. canina* genotypes were placed in one group, most of the genotypes from the Khalkhal region, were assigned to the second structure. As maintained in Figure 4, genotypes 11 and 12, as well as 21, 22 and 23 were located in the first group. However, some of the genotypes such as; N2, 10, 12, 21 and 22 showed a complex genetic structure. According to these results, there were two different groups. Most genotypes of Namin and Nir were assigned to one group and most of the khalkhal genotypes were located in a separate group. Population differentiation can also be associated with specific habitat characteristics, geographic barriers or long distances between populations (Kocovsky *et al.,* 2013). It is also argued that most of the samples have little genetic mixing. One of the important reasons for this grouping is related to the climatic conditions and geographical separation of the two regions mentioned. This genetic difference and genetic background can be considered as a valuable genetic source for breeding programs. Also, even though there were similarities between Namin and Nir genotypes but their geographic areas are different, it exhibited that the genotypes may have the same origin. When genotypes or cultivars from different regions fall into one group, it may indicate that they share the same genetic origin (Besnard *et al.,* 2001b; Sarri *et al.,* 2006). This may have happened because of plant transmissions by humans or genetic flow and displacement by natural factors (Percifield *et al.,* 2007).

The obtained results indicated that the *R. canina* genotypes investigated in this study have a wide genetic

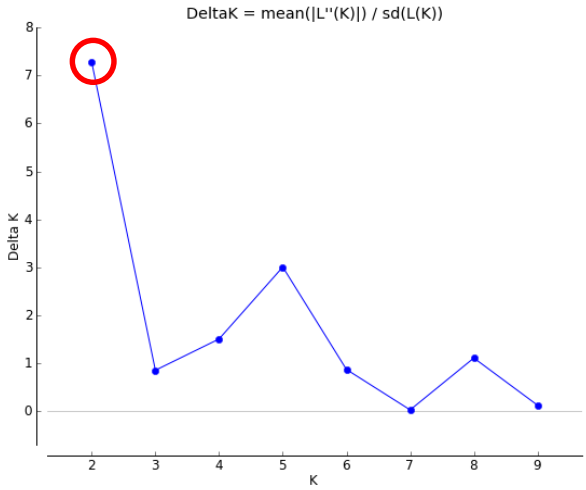

Figure 3. The subgroups obtained using the Structure harvester. The vertical and horizontal axis shows value of the *ΔK* and number of sub-populations, respectively.

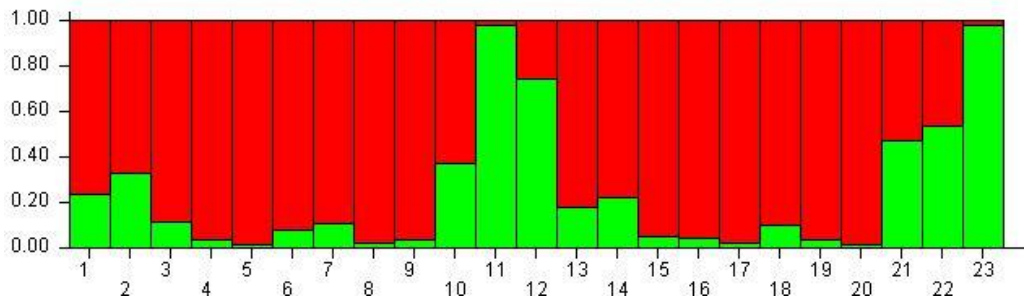

Figure 4. The BAPS analyses of 23 *R.canina* genotypes each vertical bar represent one individual genotype. According to the K=2 model, *R. canina* germplasm structured into two gene pools.

diversity (H index was between 0.14 -0.45). High genetic variation was found mainly within populations, which may have been caused by the outcrossing phenomenon. Cluster analysis using the WARD method grouped the population from Ardebil province according to its geographical origin. Towards a better understanding of the genetic diversity of *R. canina* genotypes, future studies should focus on a larger number of populations and accessions collected from more geographical regions. Finally, the results of this work showed that ISSR markers separated genotypes based on geographic regions and by determining the genetic distance between genotypes. Selection of the appropriate parents to be used in artificial crosses is one of the main decisions faced by plant breeders. The best way to select parents is the use of genotypes with more genetic distance, to produce superior recombinant genotypes. In this regard, genotypes from Khalkhal and Namin could yield superior hybrids.

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